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Risk assessment of ZnO nanoparticles revelation on erythrocyte membrane

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Abstract

Rapid developments in the cosmetics, food and drug applications Zinc oxide (ZnO) nano materials have resulted in concerns related to the risk of overexposure of human blood. The rationale of this study was to systematically research the influence of nano-ZnO on erythrocyte. The ZnO nanoparticle was prepared by sol-gel method with size of 40 ± 5 nm. The erythrocytes treated with nano-ZnO underwent abnormal sedimentation, aggregation along with dose dependent haemolysis. The ghost cells were investigated by using ultrathin cell section in the case under nano-ZnO. The RBC membrane destruction further confirmed by malondialdehyde (MDA) quantification. The results imply better understanding of the interaction between erythrocytes and nano-ZnO and will help in assessing the risk of nanomaterial toxicity in blood that may have potential toxicity to human being health. The following results suggest that the risk assessment of nano-materials and nano-products should be carried out prior to their wide application although they hold much more inconceivable and remarkable advantages.

Keywords: Zinc oxide nanoparticles. Erythrocyte, Nanotoxicity

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Introduction

Nanotechnology is now making headway in different spheres of human lives. This is leading to a diverse array of products with applications in diagnosis, drug delivery, food industry, paints, electronics, sports, environmental cleanup, cosmetics, sunscreens, etc [1]. However, recent studies also had suggested that nano-materials with their tiny size (0.1–100 nm) could easily enter into human body. The small size and high surface-volume ratio endow them with an active group or intrinsic toxicity. Therefore, it is necessary to understand and assess the potential toxicity of engineered nanomaterials to avoid their adverse effects on environmental and human health [2].

Among the varieties of engineered nanoparticles being used today, zinc oxide (ZnO) nanoparticles are one of the most widely used in consumer products. They are extensively used in cosmetics and sunscreens because of their efficient UV absorption properties without scattering the visible light. This makes them transparent and more aesthetically acceptable as compared to their bulk counterpart [3]. ZnO nanoparticles are being used in the food industry as additives and in packaging due to their antimicrobial properties [4]. They are also being explored for their potential use as fungicides in agriculture and as anticancer drugs and imaging in biomedical applications [5].

The increased production and use of ZnO nanoparticles enhances the probability of exposure in occupational and environmental settings. This has culminated in some studies investigating the toxicity of zinc oxide nanoparticles in different biological systems such as bacteria and mammalian cells. In mammalian cells, the toxic effects of ZnO nanoparticles such as

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membrane injury, inflammatory response, DNA damage and apoptosis have been demonstrated [6]. As we know, nano-ZnO has to travel along the body within blood before arriving at target organs. So the influence of nano-ZnO on cells in blood should be paid more attention to for safety application. Although playing a vital role in carrying oxygen from lungs to tissues or organs to meet metabolic needs, erythrocyte, dominant (99%) cell in the blood, is vulnerable to toxicity with deformation, agglutination and membrane damage [7,8]. So it is necessary and urgent to assess the risk of nano-ZnO on erythrocyte.

In the present study, we systematically research effects of nano-ZnO on erythrocyte. Firstly the influence of nano-ZnO on erythrocyte sedimentation was detected. Secondly, hemagglutination induced by nano-ZnO was studied by optical microscopy. Finally, as ghost cell was found in some cases, hemolysis was investigated under nano-ZnO at different concentrations. Furthermore the mechanism of such adverse effects was studied by using ultra-thin cell section with transmission electron microscope (TEM) and by using oxidative stress theory with malondialdehyde (MDA) quantified in the culture fluid of erythrocyte under nano-ZnO.

Materials and methods

Zinc chloride ($ZnCl_2$), zinc nitrate ($Zn(NO_3)_2$) and sodium hydroxide (NaOH), all of analytical grade were purchased from Sigma–Aldrich used as precursors for the synthesis. ZnO particles were diluted by phosphate buffered solution (PBS, pH 7.4) and sonicated for 5 min prior to incubation with erythrocytes in order to avoid aggregation and stored at 4°C. Male white rabbits, weighed 2.3 kg and approximately 6 months of age. The animal was housed in stainless-steel wire-mesh cages in animal rooms maintained at 20 °C, and 40– 60% relative humidity. After acclimation to the laboratory for at least 3 days, the blood sample was obtained by Marginal ear vein with a syringe (3 ml) pre-treated by 1% heparin after anesthetized by intraperitoneal injection with sodium pento-barbital 40 mg/kg of body weight. Erythrocyte suspension was washed three times by isosmotic PBS (pH 7.4) and then diluted in washing solution at the concentration of 1×10^6 cells/ml and stored in 4 °C for not more than 24h.

Preparation of ZnO nanoparticles by Sol-Gel processing

The production unit of ZnO nanocrystals (nano-ZnO) consisted basically of a reactor with heating. The NaOH was dissolved in distilled water at a concentration of 1.0 M. The resulting solution was heated under constant stirring at the desired reaction temperature (50°C). After obtaining the desired temperature, a solution of $ZnCl_2$ 0.5 M and another of $Zn(NO_3)_2$ 0.5 M were slowly added (dripping for 26 min) into the reactor. This procedure was performed under constant stirring and the reaction temperature was maintained at the desired value. The dripping of the $ZnCl_2$ and $Zn(NO_3)_2$ solutions in an aqueous alkaline solution results in immediate precipitation of ZnO, and the colour of the suspension changes from transparent to white. After completing dripping, each solution remained agitated for a period of two hours, maintaining the desired temperature. The material formed in the reactor was filtered, washed several times with deionised water and dried in a vacuum oven at a maximum temperature of 70°C for several hours [9].

Size of ZnO observed by TEM

Particle size distribution was characterized by high resolution transmission electron microscopy (HRTEM) using a PHILIPS (CM200). All TEM measurements were performed on 100 µg/ml stock suspension. For each measurement, 15 mL of the colloidal solution was placed onto a carbon coated copper grid and allowed to dry for 10 min. Images were recorded and particle size distribution was determined by Image J software package. Approximately 100 nanoparticles were measured in random fields of view of three images to calculate mean particle size [9].

Erythrocyte sedimentation and aggregation

One hundred microliters of erythrocyte suspension (1×10^6 cells/ml) and nano-ZnO at the final doses (100 and 200 µg /ml) were incubated together in EP vials for 1 h at the temperature 4°C. The erythrocyte treated with PBS (pH 7.4) was taken as the control. The erythrocyte sedimentation was investigated and recorded by using camera. The erythrocyte aggregation of the control group, the nano- ZnO group (100 µg /ml) were observed by using light microscope [10].

Ultra-thin erythrocyte section observed by TEM

One hundred microliters of the erythrocyte suspension (1×10^6 cells/ml) and nano-ZnO at the final dose (100 µg /ml) were incubated in EP vials at the temperature 4°C for 12 h. The erythrocyte treated with PBS (pH 7.4) was taken as the control. For TEM analysis, each erythrocyte-ZnO mixture was centrifuged at 1000 rpm for 10 min. The pellet was washed three times by PBS (pH 7.4) and fixed with 2.0% glutaraldehyde in washing solution for 12 h. After washed as above, the erythrocytes were post-fixed for 4 h with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, and then were dehydrated in a series of ethanol solution (50%, 70%, 90%, 95%, 100% and 100%). Finally the erythrocytes were incubated in the mix-ture of LR White and ethanol (2:1) for 2 h and (1:1) for 12 h at 4°C, and embedded in pure LR White for 72 h at 37 LC. Ultra-thin cell section (70 nm) was cut and transferred on 200-mesh grids, stained with uranyl acetate, counter-stained with lead citrate and ultimately observed with TEM [11].

Haemolysis under nano-ZnO

One hundred microliters of erythrocyte suspension (1×10^6 cells/ml) and nano-ZnO at different doses (100, 200 µg/ml) were incubated together for 1 h at the 4°C. The Triton X 100 (1% v/v) were used as a standard. The mixtures were centrifuged at 1000rpm for 10 min. Each sample was repeated four times. The degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm. The absorbance of the control group was used as the blank. The supernatant was diluted if necessary to keep in this range the concentration of the tested solution. The absorbance value accounted for the amount of hemoglobin release induced by nano-ZnO [12].

Measurement of erythrocyte lipid peroxidation

One hundred microliters of the erythrocyte suspension (1×10^6 cells/ml) was cultured with nano- ZnO at final doses (100 and 200µg/ml) for 12 h at 37°C, the total experiment volume was 200µl. The erythrocyte treated with the PBS (pH 7.4) was taken as the control. One hundred microliters culture fluid was carefully removed for MDA analysis according to the MDA Detection Kit operating manual [13].

Statistical analysis

The data were treated and analyzed by software graph pad prism version 5. The value was presented with mean ± SD deviation. Both the one-way analysis of variance (one-way ANOVA) test was performed to compare the data. The following indicate significant differences from control: *$p < 0.01$, **$p < 0.005$.

Results

Preparation of ZnO nanoparticles

ZnO nanoparticles with wurtzite structure were obtained from a sol-gel method quickly and easily, using zinc chloride and nitrate as precursors. The smallest crystallite size is obtained using chloride as a precursor in the synthesis temperature 50°C.

Size of nano-ZnO observed by TEM

Images of high resolution transmission electron microscopy (HRTEM) show that zinc oxide nanoparticles were obtained with sizes close to 40 nm [Figure. 1a]. The image (Figure. 1b) shows the basal plane of the wurtzite structure, which has a higher growth rate.

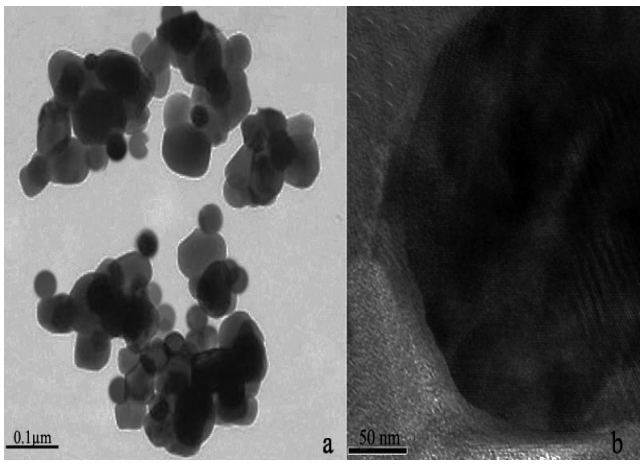


Figure 1. Size of TiO2 observed by TEM. (a) The size of nano-ZnO was about 40 ± 5 nm and bar scale is 100 nm and (b) high resolution TEM image of the wurtzite structure for the condition ZnO.

Erythrocyte sedimentation and aggregation

Erythrocyte sedimentation rate (ESR) is to observe the height to which the erythrocytes fall in a given time interval, usually 1 h. In general, the sedimentation under gravity can monitor the dramatic abnormal alteration in shape and membrane of erythrocyte induced by diverse toxicants.

Erythrocyte sedimentation and aggregation the erythrocytes under nano-TiO_2 were obviously agglutinated, large-partly accumulating to the bottom and a few still suspending in the top, totally distinguished from the control. Meanwhile the ESR of the nano-ZnO tubes more than that of control. Increasing of ESR can be reasoned by that those erythrocytes under nano-ZnO settled down more quickly than the control. This result implies that nano-ZnO have the potential influence on erythrocyte shape. Furthermore, the apparent haemolysis (red color) in the supernatant of nano-ZnO groups also suggests that the integrity of erythrocyte membrane has been damaged by nano-ZnO.

The erythrocytes morphology were studied by photo microscope, compared to the control the erythrocytes coated with the nano-ZnO particles, exhibited abnormal shape with the surface herniations, which were referred to acanthocytic erythrocytes. The amount of acanthocytic erythrocytes may relate to the surface properties of the nano-ZnO and its high surface to mass ratio.

Ultra-thin erythrocyte section observed by TEM

TEM is used further to obtain the information about the entering and localization of nano-ZnO in erythrocyte. As shown in [Figure 2] nano-ZnO aggregated was found to be attached along to the membrane of erythrocyte. The high amplification figures [Figure. 2a] indicated that only a few was investigated within the erythrocytes. At higher magnification the aggregated nano-ZnO was seen to insert into erythrocyte by trans-membrane [Figure. 2b]. The nano-ZnO was clearly seen to be located around the trace of erythrocyte membrane, where the bilayer membrane partly loomed over the periphery of ghost cells. The ghost cells, as the severe adverse impact of nano-ZnO on erythrocyte, suggest nano-ZnO have damaged the integrality of erythrocyte and hence the contents leak to the outside of erythrocyte through the breakage.

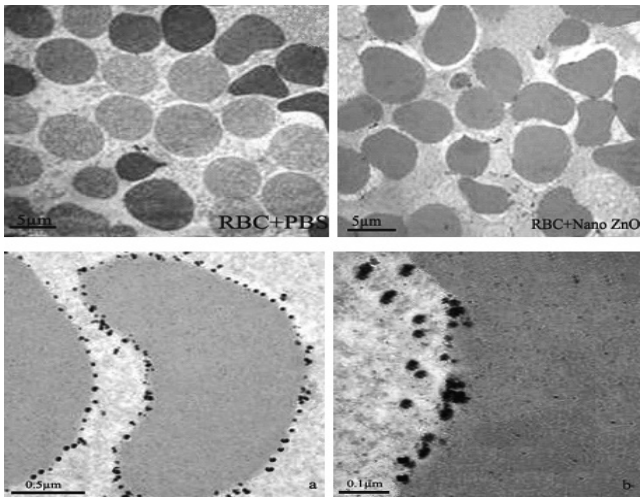


Figure 2. Ultra-thin section of erythrocyte under nano-ZnO analyzed by TEM. Erythrocyte + PBS, erythrocyte + nano-ZnO (100 μg/ml, (a&b) erythrocyte + nano-ZnO (100μg/ml) at higher magnification. Nano-ZnO aggregated was found to be attached along to the membrane of erythrocyte [Figure. 3a]. At higher magnification the nano-ZnO was seen to insert into the membrane [Figure. 3b].

Haemolysis under nano-ZnO

Ghost cells resulted from the breakage of erythrocyte membrane exposed to nano-ZnO. Another result of the breakage was haemolysis, which happened when the content escaped from the inner to the outer of erythrocyte. The haemolysis increased, sharply under nano-ZnO with the dose rising. The dose dependent hemolysis was showed the standard triton X 100 (1%v/v) showed 85% haemolysis, for nano-ZnO 100, 200μg/ml induce the haemolysis 62 and 75% respectively.

Lipid peroxidation of erythrocyte membrane under nano-ZnO

Reactive oxygen species (ROS) consist of superoxide radical, hydrogen peroxide (H_2O_2), single atomic oxygen, hydroxyl radical, lipid peroxidation, etc. Mitochondria are the main source (98% in total) that generates ROS. These ROS are quenched by some enzyme such as SOD and catalase in cell. Erythrocytes are incapable to produce SOD and catalase, hence are vulnerable to the extraneous toxicants. And the cell membrane may be easily damaged during lipid peroxidation. Lipid peroxides, reactive products of lipid peroxidation, has deleterious effects on the cell membrane. MDA, the major product of lipid

peroxidation has been extensively used as an index for lipid peroxidation and as a marker for oxidative stress. The reaction of MDA with TBA has been widely adopted as a sensitive assay method for lipid peroxidation.

The result [Figure. 3] exhibits that lipid peroxidation increases sharply in the dose dependent way under nano-ZnO. Increased MDA reconfirms that the more the contact is, the more severe the lipid peroxidation. The result also suggests that erythrocyte membrane is damaged by MDA besides the nano-ZnO insertion.

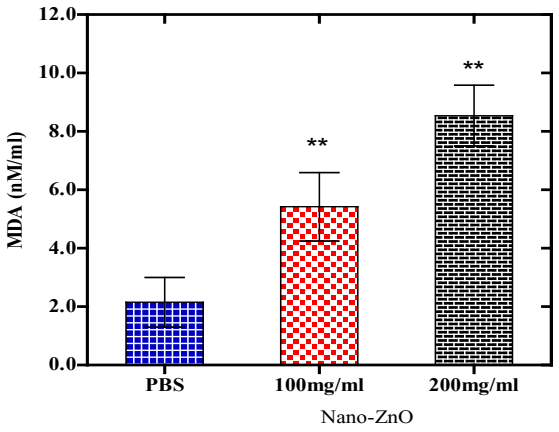


Figure 3. MDA under nano-ZnO. Erythrocytes treated with PBS as the control group, with nano-ZnO (100 and 200 μg/ml), respectively. The data was conducted by one-way ANOVA. The following indicate significant differences from control: *p < 0.01, **p < 0.005 (relative percentage of control).

Discussion

As we know, ultrafine particles in air are defined as particles in size less than 100 nm, compared with fine particles (>100 nm). The size falls into the category of nano-scale. Nanotoxicity is a new term and many data about nanotoxicity are firstly derived from epidemiological studies about ultrafine particles such as pneumoconiosis. In addition, generally accepted nomenclature of nano-sphere includes nanoparticles, ultrafine particles and quantum dots [14].

Risk assessment of nanomaterials is getting important in diverse areas and applications. However, the requested information and data are far below and there have been many things to examine and study these impacts on cells, tissues, and organs as well as the mechanism of such action [15]. In general, any hazard influence of these chemicals on cells in blood should be avoided if possible. As for nanoparticles, when they are introduced intentionally or unintentionally into human body the risk on cells, particularly on erythrocytes, must be conducted for safety [16].

In this study series of experiments, we can conclude that nano-ZnO has gravely deleterious effect on erythrocyte in a dose-dependent way *in vitro*. Firstly, the nano-ZnO was adsorbed to the erythrocyte membrane since their high surface-volume ratio, one of nano-ZnO characteristics, is known to facilitate them adsorbing. Once the majority surface was covered by nano-ZnO erythrocyte showed a tendency to be aggregation because the membrane-bound nano-ZnO deformed erythrocyte and hence decreased the repulsion among erythrocytes. RBC aggregation was successfully explained with a cell depletion layer model in which specific proteins were present at extremely low concentrations near the cell membrane [17, 18]. When nanomaterials attach to the cell membrane, the depletion layer would be disrupted and the corresponding RBC aggregation would also be affected [19, 20]. This was reconfirmed by the next result that the morphological change from bi-concave shape to modification had aggravated hemoagglutination. Secondly, as we known, the erythrocyte membrane is composed of a lipid bi-layer primarily with protein embedded in, which keeps the membrane in dynamic equilibrium between fluidity and solidity [21, 22]. With the attachment tighter, the aggregated nano-ZnO damaged the membrane by inserting into lipid bi-layer in fluidity state and also by locating within the plasma. In addition, nano-ZnO could produce free radicals (i.e., reactive species of molecules) and exert a strong oxidizing ability. Erythrocyte is extremely vulnerable to oxidative damage because of the high poly-unsaturated fatty acid content in the membranes. MDA is not only the well-characterized product of the lipid peroxidation of erythrocytes but also one highly reactive and bi-functional molecule to impair a variety of membrane-related functions through cross-linking erythrocyte phospholipids and proteins [23]. The high active molecule ultimately leads to the decrease of erythrocyte survival (hemolysis) and generation of ghost cells.

Conclusions

The mechanism of nano ZnO toxicity in erythrocyte membrane following (a) the attachment of nanomaterial

around erythrocyte changes the surface properties and ultimately lead to hemoagglutination (b) the content leak to the outside of erythrocyte through the breakage induced by both the nano-ZnO trans-membrane and the oxidative stress under the influence of nano-ZnO free radicals. Our results finding that the risk assessment of nano-materials and nano-products should be carried out prior to their wide application although they hold much more inconceivable and remarkable advantages.

Conflict of Interest

Nil

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